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DETERMINATION OF 5-BROMO-2'-DEOXYURIDINE (BrdU) IN WELL WATER BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

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A liquid chromatographic method has been developed for the analysis of 5-Bromo-2'-deoxyuridine (BrdU) in well water. The samples are injected directly onto a high performance liquid chromatograph (HPLC) without further sample preparation. Separation of BrdU from possible interferences found in well water was achieved by using a C-18 column and a mobile phase containing water and methanol. A programmable ultraviolet (UV) detector was used to monitor the effluent for BrdU. HPLC provides a rapid and reproducible method for the analysis of BrdU.

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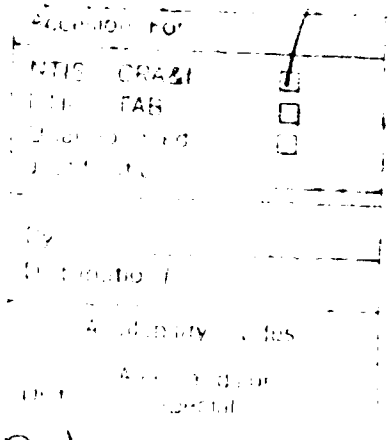

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DETERMINATION OF 5-BROMO-2'-DEOXY-URIDINE (BrdU) IN WELL WATER BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

INTRODUCTION AND OBJECTIVES

The U.S. Army Biomedical Research and Development Laboratory has been involved in the use of 5-bromo-2'-deoxyuridine (BrdU) as a label of DNA replication in Japanese medaka (Oryzias latipes). Because tumor cells generally have a higher rate of cell proliferation and therefore a higher rate of DNA replication[1], BrdU is being examined as a means to trace cell division in medaka. The ability to track DNA replication aids in the determination of the early biological effects of potentially hazardous chemicals, providing information on the development of cancer. BrdU has been analyzed in biological fluids[1-2] by HPLC. These methods have been modified to determine the level of BrdU in the well water used to expose the Japanese medaka to BrdU.

BrdU is a thermally labile compound that decomposes at 191-194 ^{o}C. This property prohibits the use of gas chromatography as an analytical technique. The ability of high performance liquid chromatography (HPLC) to provide a method of separation at temperatures below the point where the thermal decomposition of BrdU occurs makes HPLC the preferred method. The method was developed for determining BrdU in well water at levels of 10 to 500 mg/L. Direct injection and a relatively short run time allow for the rapid analysis of well water samples. HPLC provides an accurate and precise method for the determination of BrdU in well water samples.

METHODS AND MATERIALS

INSTRUMENTAL

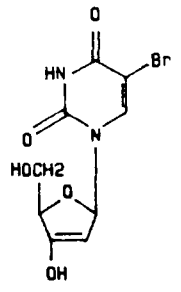
A Hewlett Packard 1050 series HPLC equipped with variable wavelength detector, autosampler, and Hewlett Packard 3396A integrator (Hewlett Packard, Avondale, PA) was used throughout the study. The UV detector was set at 277 nm (0.2 aufs). The solvent delivery system was programmed to deliver 20 percent methanol/water eluent at a flow rate of 1.5 mL/minute. A Supelcosil™ LC-18 column (25 cm x 4.6 mm i.d., 5 micron particle size, Supelco, Bellefonte, PA) was used for the separation of BrdU. The injection volume was 1 microliter. An Endocal™ RTE-9DD refrigerated circulating bath (Neslab Instruments, Inc.,Portsmouth, NH) set at 25 ^{o}C was used for the determination of the stability of BrdU in well water.

REAGENTS AND MATERIALS

High purity grade methanol (Burdick & Jackson, Muskegon, MI) was used without further purification. BrdU was purchased from Sigma Chemical Company, St. Louis, MO. Water for HPLC was purified with a Barnsted Nanopure™ water purification system (Barnsted/Thermolyne corp., Dubuque, IA.

The structural formula and other pertinent data for BrdU follows:

CAS Registry Number: 59-14-3
RTECHS Reference Number: YU7350000
M.W.: 307.13
Chemical Formula: C9-H11-Br-N2-O5
Description: White Powder
Melting Point: 376-381 ^{o}F (191-194 ^{o}C) (Decomposes)
Structure:



5-BROMO-2'-DEOXYURIDINE

Synonyms: BDU; 5-BDU; BRDU; Bromodeoxyuridine; 5-Bromodeoxyuridine; 5-Bromodeoxyuridine; 5-Bromo-2-deoxyuridine; 5-Bromo-2'-deoxyuridine; Bromouracil deoxyriboside; 5-Bromouracil deoxyriboside; 5-Bromouracil-2-deoxyriboside; Broxuridine; BRUDR; BUDR; 5BUDR

SAMPLE PREPARATION

Aqueous solutions were received in glass vials. The samples were transferred to 2-mL autosampler vials with Teflon™ crimp seals and analyzed immediately. Each sample containing BrdU in excess of 500 mg/L was diluted in a volumetric flask to obtain a concentration between 10 mg/L and 500 mg/L.

PREPARATION OF STOCK AND STANDARDS

A BrdU stock solution was prepared by dissolving 0.100 g of BrdU in 100 mL of reagent grade water to give the final concentration of 1000 mg/L. Fresh working standards were prepared by diluting the stock standard to give 5, 50, 250, and 600 mg/L solutions. Both stock and standard solutions were prepared fresh each day of the analysis.

CALCULATIONS

Peak areas for all working standards are plotted against their concentrations and linear regression analysis was performed on the data. This determination was prepared daily and used to determine BrdU concentrations in well water.

RESULTS AND DISCUSSION

A chromatogram of a well water sample containing 50 mg/L is shown in figure 1. The mobile phase and instrumental conditions are listed in the methods section. The retention time of each peak is the identifier for the analyst. Any deviation of $\pm$ 0.2 minutes from this time is considered suspect to examination. The detection limit is defined as the lowest concentration that can be reproduced ten times with a relative standard deviation of not more than 10 percent. A standard deviation of not more than 10 percent is deemed an acceptable limit. The detection limit for this method is 1.77 mg/L with a relative standard deviation of 8.02 percent and a 95 percent confidence limit of 1.87 mg/L (upper limit) and 1.67 mg/L (lower limit). This detection limit can be extended downward, if required, by increasing the volume of sample injected. Prior to analysis, a standard solution containing a known concentration of BrdU is injected onto the HPLC. Peak areas for each working standard were plotted against its concentration to obtain a standard curve as shown in figure 2. Samples of well water containing BrdU in concentrations ranging from 10 mg/L to 500 mg/L are analyzed by direct injection onto a HPLC equipped with a UV spectrophotometer detector. The concentrations were determined by linear regression analysis.

Precision of the method was determined by injecting five samples three times on three separate days. Mean, standard deviation, and relative standard deviation were calculated at the high and low concentrations in the range of interest (500 mg/L and 10 mg/L). The precision data is shown in Table 1.

Table 1. BrdU Precision data.

LOW LEVEL

DAY	MEAN (mg/L)	S.D.	R.S.D. (%)
1	9.81	0.010	0.1
2	9.94	0.311	3.1
3	10.06	0.447	4.4
AVG.		0.256	2.5

HIGH LEVEL

1	493	14.25	2.9
2	507	6.19	1.1
3	499	10.60	2.1
AVG.		10.35	2.0

The accuracy of the method is better defined by percent recovery. This was determined by taking five aliquots of a sample containing 9 mL of a well water sample with 1 mL of the appropriate BrdU standard. The aliquots were then analyzed to obtain a mean, standard deviation, relative standard deviation and percent recovery. This was performed at the high and low concentrations in the range of interest (500 mg/L and 10 mg/L). The data for this determination is shown in table 2.

Table 2. BrdU accuracy data.

LOW LEVEL

DAY	AMOUNT ADDED (mg/L)	AMOUNT RECOVERED (mg/L)	S.D.	RSD (%)	REC. (%)
1	11.0	11.0	0.11	1.0	100.0
2	11.2	10.8	0.38	3.5	96.4
3	11.1	11.5	0.99	8.6	103.6
AVG.			0.49	4.4	100.0

HIGH LEVEL

1	510	513	2.12	0.4	100.6
2	521	523	2.73	0.5	100.4
3	515	516	1.58	0.3	100.2
AVG.			2.22	0.4	100.4

STANDARD CURVE DATA

DAY	SLOPE	R^2 VALUE	Y-INTERCEPT
1	439	0.9999	101
2	438	0.9999	241
3	439	0.9999	263

Possible matrix interferences from well water were determined by the serial dilution of a well water sample with reagent grade water. A stock solution of 500 mg/L BrdU in well water was prepared. Five mL of the well water stock containing BrdU were diluted with 5 mL of reagent grade water. Then 1 mL of the well water stock containing BrdU was diluted with 9 mL of reagent grade water. Three aliquots of stock and each dilution were analyzed to obtain a mean, standard deviation, relative standard deviation and percent recovery. The data for possible interferences is shown in table 3.

Table 3. BrdU interference data

AMOUNT ADDED (mg/L)	AMOUNT RECOVERED (mg/L)	S.D.	RSD (%)	REC. (%)
500	500	2.00	0.4	100.0
250	250	1.73	0.7	100.0
50.0	50.5	0.21	0.4	101.0

The stability of BrdU was determined by injecting a spiked well water sample at regular intervals over a seven-day period. The sample was stirred and kept in a sealed jacketed flask held at 25.0 ^{o}C. Each day a new stock solution of approximately 500 mg/L was prepared to produce a fresh set of standard solutions. A standard curve was plotted for each day. From the peak area the concentration was determined, this data is shown in table 4. The concentrations were plotted versus time over the 168 hour period as shown in figure 3.

Table 4. Data for BrdU Seven Day Stability Study.

Time (Hours)	Concentration (mg/L)	Time (Hours)	Concentration (mg/L)
0	112.1	98.5	107.3
0.17	113.0	99.0	107.2
0.33	111.8	99.5	107.1
0.5	112.1	100.0	108.0
0.67	112.3	100.5	107.7
0.83	113.0	101.0	107.7
1.0	112.9	101.5	106.8
1.5	112.0	118.0	108.0
2.0	112.4	118.5	109.9
2.5	111.9	119.0	108.3
3.0	110.8	120.0	108.6
3.5	112.2	120.5	108.9
4.0	112.2	121.0	109.2
4.5	111.7	121.5	108.4
5.0	112.4	122.0	109.1
5.5	111.5	122.5	108.2
23.0	109.4	123.0	108.1
23.5	110.3	123.5	109.0
24.5	109.9	124.0	108.7
25.0	109.7	124.5	108.7
25.5	109.7	125.0	109.0
26.0	110.5	142.5	112.8
26.5	109.5	143.0	111.8
27.0	110.4	143.5	112.8
27.5	110.2	144.0	112.8
28.0	110.0	144.5	112.3
28.5	110.3	145.0	111.6
29.0	110.1	145.5	112.9
29.5	110.1	146.0	111.4
47.0	111.2	146.5	112.6
47.5	110.1	147.0	112.8
48.0	110.4	147.5	111.6
48.5	110.1	148.0	112.2
75.0	107.0	148.5	112.2
75.5	107.3	149.0	112.2
76.0	107.4	149.5	111.5
76.5	108.2	166.5	108.4
94.5	108.2	167.0	108.5
95.0	106.5	167.5	106.9
96.0	107.6	168.0	107.2
96.5	108.1	168.0	107.7
97.0	107.6	168.0	107.9
97.5	107.5	168.0	107.3
98.0	107.2		

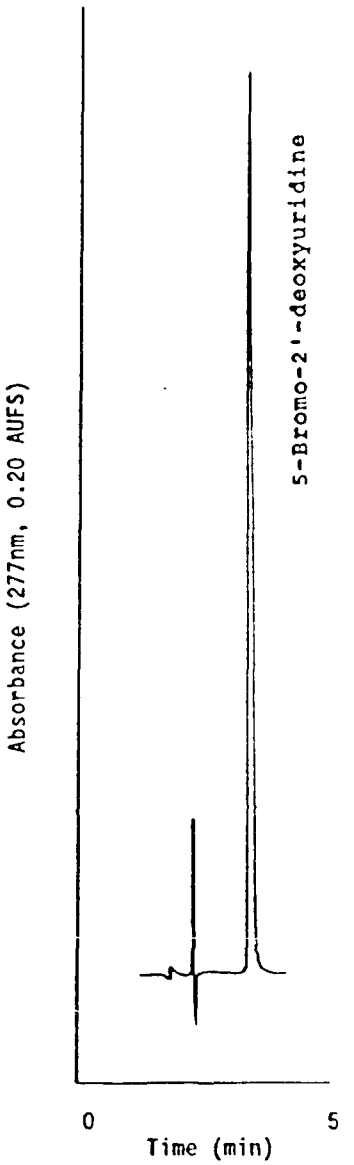


Figure 1. HPLC chromatogram of a well water sample containing 50 mg/L BrdU.

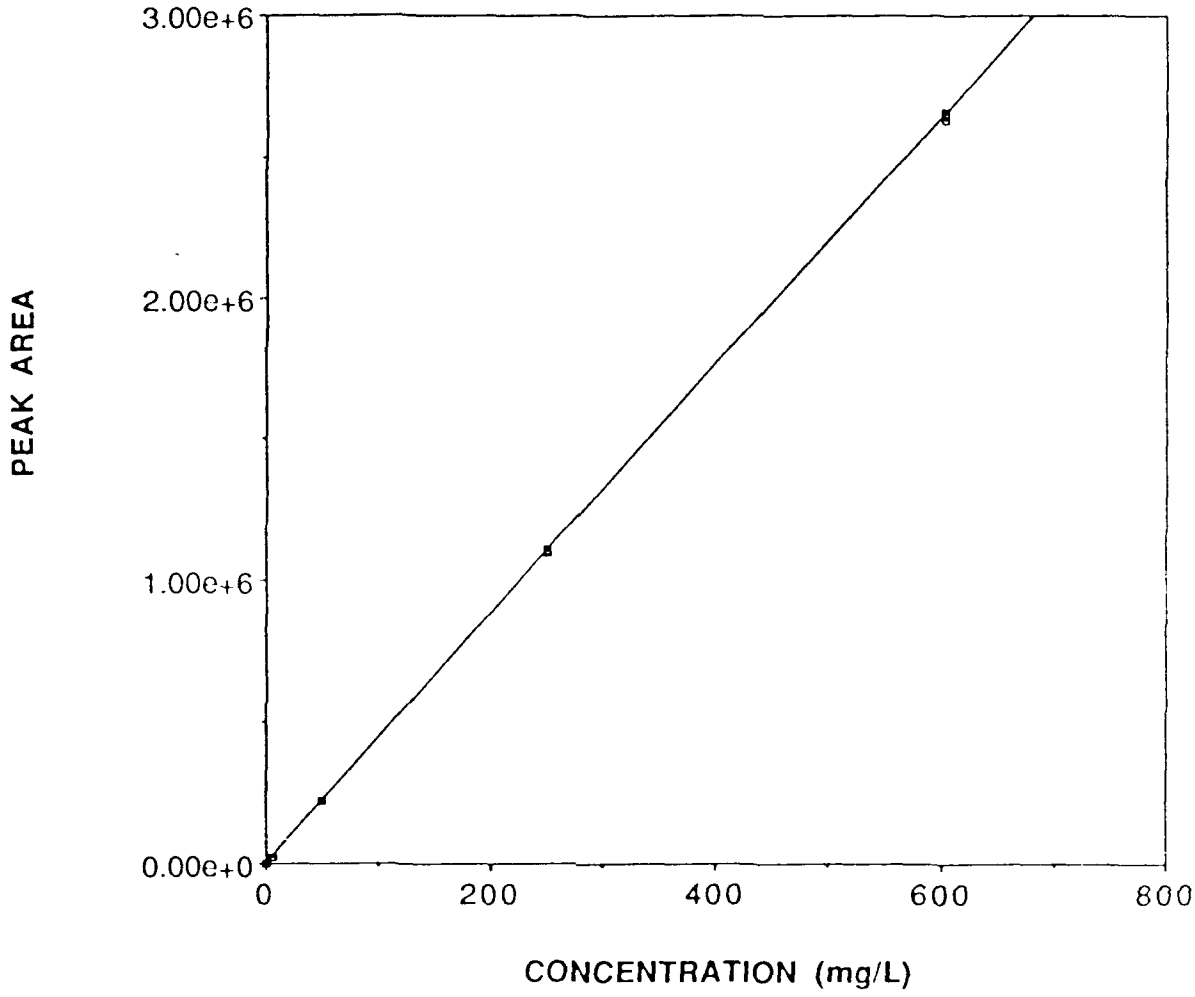


Figure 2. Plot of BrdU standard curve.

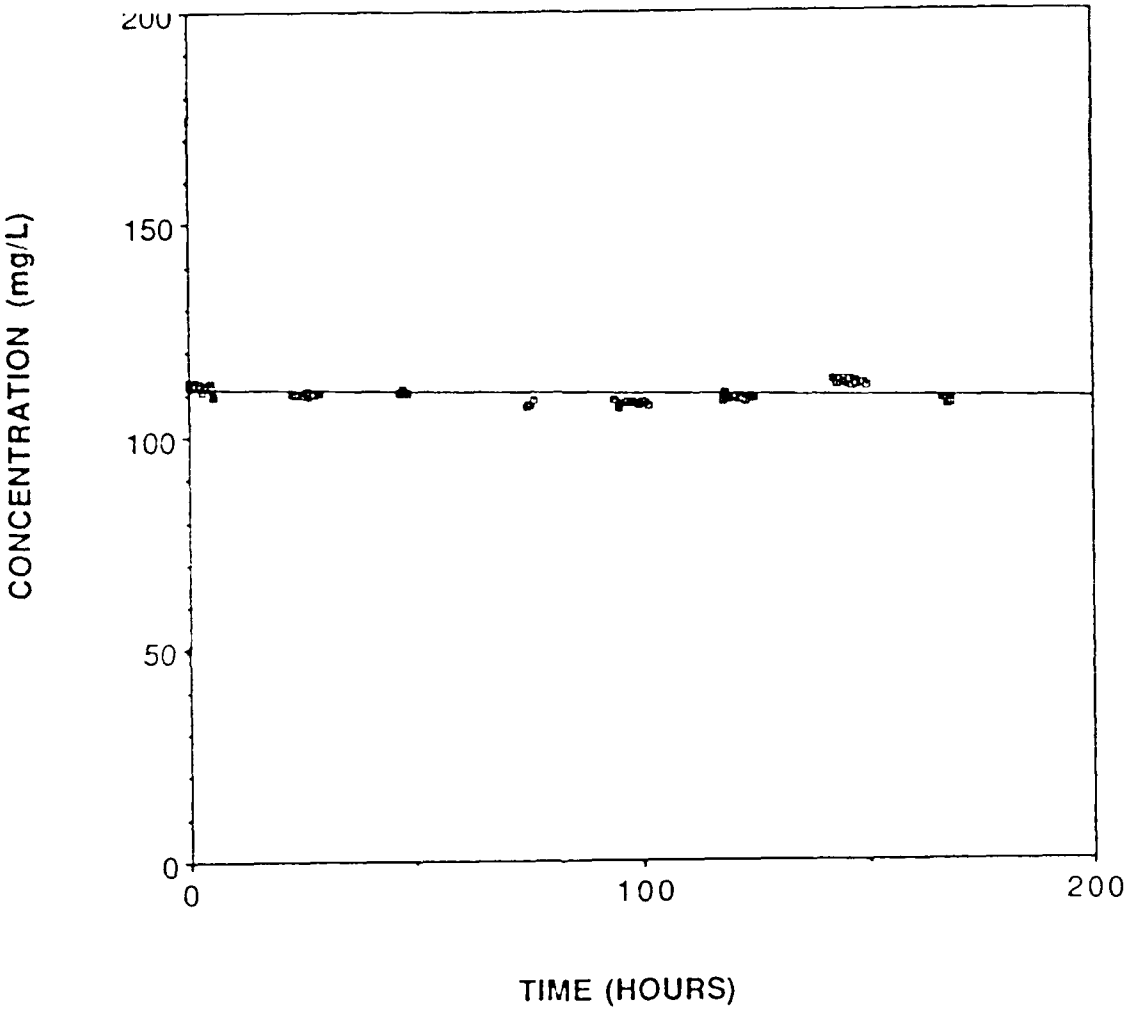


Figure 3. Plot of BrdU stability data versus time.

CONCLUSION

HPLC provides an accurate and precise method for the determination of BrdU in well water. The instrumental conditions described in the instrumental section provide a method for the separation of BrdU from interferences in well water. Direct injection of the sample and relatively short run time allow for the rapid analysis of a large number of BrdU samples in a relatively short period of time. Good sensitivity was provided by monitoring the column effluent at 277 nm. The ability of HPLC to analyze thermally labile compounds allows for the analysis of BrdU without thermal decomposition that would occur if gas chromatography was used.

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